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Cultivation-Independent Detection of Autotrophic Hydrogen-Oxidizing Bacteria by DNA Stable-Isotope Probing[▽]

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Knallgas bacteria are a physiologically defined group that is primarily studied using cultivation-dependent techniques. Given that current cultivation techniques fail to grow most bacteria, cultivation-independent techniques that selectively detect and identify knallgas bacteria will improve our ability to study their diversity and distribution. We used stable-isotope probing (SIP) to identify knallgas bacteria in rhizosphere soil of legumes and in a microbial mat from Obsidian Pool in Yellowstone National Park. When samples were incubated in the dark, incorporation of $^{13}CO_2$ was H_2 dependent. SIP enabled the detection of knallgas bacteria that were not detected by cultivation, and the majority of bacteria identified in the rhizosphere soils were betaproteobacteria predominantly related to genera previously known to oxidize hydrogen. Bacteria in soil grew on hydrogen at concentrations as low as 100 ppm. A *hydB* homolog encoding a putative high-affinity NiFe hydrogenase was amplified from ^{13}C-labeled DNA from both vetch and clover rhizosphere soil. The results indicate that knallgas bacteria can be detected by SIP and populations that respond to different H_2 concentrations can be distinguished. The methods described here should be applicable to a variety of ecosystems and will enable the discovery of additional knallgas bacteria that are resistant to cultivation.

Bacteria that grow autotrophically using H_2 as an electron donor and O_2 as an electron acceptor (referred to as knallgas bacteria) are a physiologically defined group with representatives in the *Proteobacteria*, *Aquificales*, *Actinobacteria*, and *Firmicutes* (3, 42, 44). They can be isolated from a wide range of ecosystems, including soil, sediment, seawater, thermophilic compost, and geothermal sites (2, 3), but their ecological role as H_2 oxidizers is poorly understood. Soil is the largest sink of tropospheric hydrogen, which is present at a mixing ratio of 550 ppb, but knallgas bacteria typically have low-affinity hydrogenase activity ($K_m > 800$ nM), with a threshold for H_2 uptake ranging from 1 to 200 ppm, and thus are unable to oxidize tropospheric H_2 (10). The oxidation of tropospheric hydrogen has been attributed primarily to abiontic hydrogenases in soil (9, 21, 46), but recently isolated strains of *Streptomyces* with a high-affinity NiFe hydrogenase (encoded by *hydB*) have been reported to oxidize tropospheric H_2 (12, 13). Although obligate chemolithotrophic H_2-oxidizing bacteria have been isolated (29, 40, 47), the widespread distribution of knallgas bacteria suggests that most knallgas bacteria grow heterotrophically when H_2 is at tropospheric concentrations and grow chemolithotrophically only when H_2 is available at higher concentrations (8).

Ecosystems that could potentially support chemolithotrophic growth of knallgas bacteria include the rhizosphere of legumes, marine surface waters, geothermal sites, and cyanobacterium-dominated microbial mats. Hydrogen is a by-product of nitrogen fixation in root nodules (45), and if the N_2-fixing symbionts are deficient in uptake hydrogenase, the H_2 is released to the surrounding soil. Dong and Layzell calculated that soil within 4 cm of N_2-fixing root nodules is exposed to H_2 at a rate of 30 to 254 nmol cm^{-3} h^{-1} (14), and both hydrogen consumption and the most probable number (MPN) of knallgas bacteria decreased exponentially with distance from root nodules (30). Knallgas bacteria in the rhizosphere may benefit the legume as well, given that H_2 enhances soil fertility (15), and H_2-oxidizing bacteria promote plant growth (36).

Less is known about the ecological roles of knallgas bacteria in other ecosystems where H_2 concentrations are above tropospheric levels. Saturation of H_2 in low-latitude surface ocean water is commonly observed (11, 22), and hydrogenase genes are abundant in marine environments (4), but almost nothing is known about knallgas bacteria and H_2 metabolism in marine environments (3). In geothermal systems, there is evidence that knallgas bacteria play a key role in primary production at temperatures greater than 70°C (50). Significant fluxes of H_2 from cyanobacterial mats have been documented (24), and H_2 exchange between N_2-fixing cyanobacteria and knallgas bacteria has long been a subject of speculation (8) but never established.

Because they are a physiologically defined group, identification of knallgas bacteria has relied primarily on cultivation. Given that current cultivation techniques fail to grow most bacteria, reliance on cultivation limits our understanding of knallgas bacteria in the natural world. Cultivation-independent techniques that selectively detect and identify knallgas bacteria will improve our ability to study their diversity and distribution. Changes in soil microbial community structure following exposure to H_2 have been detected with terminal restriction fragment length polymorphism (T-RFLP) and fluorescence *in situ* hybridization (FISH)-microscopy with group-specific probes, but the bacteria that metabolized H_2 were not identified (41, 51, 55). Primers designed to amplify hydrogenase genes have also been used to detect knallgas bacteria in soil.

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Lechner and Conrad (31) designed two PCR primer sets to detect NiFe hydrogenase genes. One pair of primers was not specific to knallgas bacteria, since they also amplified hydrogenase genes from heterotrophic N_2-fixing bacteria. The other pair was limited to bacteria closely related to *Alcaligenes eutrophus* and did not enable broad-range detection of knallgas bacteria.

Stable-isotope probing (SIP) is a cultivation-independent technique that links function and identity (39), and SIP has already been used to identify chemolithoautotrophs, including ammonia-oxidizing bacteria, iron-oxidizing bacteria, and sulfur-oxidizing bacteria and archaea (7, 18, 19, 27, 53, 54). Hydrogenotrophic methanogens have been detected by SIP, as well (33). In the present study, we expanded the application of SIP as a tool for the cultivation-independent identification of knallgas bacteria. Knallgas bacteria that incorporated $^{13}CO_2$ were identified from two rhizosphere soils with distinct H_2 uptake kinetics by generating 16S rRNA gene clone libraries from ^{13}C-labeled DNA (^{13}C-DNA). Furthermore, SIP incubations conducted under a range of H_2 concentrations distinguished among bacteria with different H_2 uptake thresholds.

MATERIALS AND METHODS

Sample collection. White clover (*Trifolium repens*) and hairy vetch (*Visia villosa*) plants on the Georgia Institute of Technology campus in Atlanta, GA, were unearthed with roots intact in soil to a depth of 5 to 8 cm. The rhizosphere soil was collected by removing bulk soil and separating the root-associated soil with a sterile spatula. Plant matter and root nodules were removed, and the rhizosphere soil was used immediately for SIP incubations.

A green microbial mat floating at the edge of Obsidian Pool in Yellowstone National Park was collected in a sterile container. The temperature of the mat ranged from 30°C in the middle to 44°C at the periphery. The mat was transported on ice to the Georgia Institute of Technology in Atlanta, GA, and SIP incubations were prepared immediately upon arrival.

Culture conditions. Knallgas bacteria were isolated by spread plating serial dilutions of collected samples on basal mineral medium (3) agar plates supplemented with 10 mM sodium bicarbonate. The plates were incubated in a sealed incubator under an H_2 concentration of 1,000 to 2,000 ppm in air. Selected colonies were streaked on R2A agar to check for purity. Isolates were tested for differential growth when transferred to fresh medium and incubated in the sealed chamber with or without added hydrogen to determine whether they grew autotrophically with hydrogen. Isolates showing increased growth when incubated under hydrogen were tested for the ability to consume hydrogen by gas chromatography-thermal conductivity detection (GC-TCD) (see below) when grown on agar slants in sealed serum bottles.

Stable-isotope probing. The SIP experiments included three treatments: one with $^{13}CO_2$ and H_2 in the headspace, a control with $^{13}CO_2$ and no H_2, and a control with $^{12}CO_2$ and H_2. The treatment with $^{13}CO_2$ without H_2 controlled for ^{13}C labeling of bacteria due to autotrophic growth with endogenous electron donors or the incorporation of inorganic carbon during heterotrophic growth (16, 23). DNA extracted from the treatment with $^{12}CO_2$ provided a control for the location of DNA not enriched with ^{13}C in CsCl gradients following isopycnic ultracentrifugation. For incubations with soil, 5 g of soil was used for the experiments with 800 ppm H_2, and 2 g was used for the experiments with lower H_2 concentrations. The clover and vetch soils had moisture contents of 47% and 40% water holding capacity. Soil was placed at the bottom of 2.1-liter flasks that were subsequently crimp sealed with a butyl rubber stopper. The flasks were flushed for 1 min with air that was passed through a 5 M NaOH solution to remove $^{12}CO_2$. Ten milliliters of $^{13}CO_2$ or $^{12}CO_2$ was injected into each flask, the overpressure was released, and H_2 was added to the desired concentration. The soil was incubated in the dark without shaking at 22 to 25°C, and the headspace H_2 concentration was monitored. The soil was removed and stored at −80°C at the end of the experiment.

SIP incubations with the microbial mat sample from Obsidian Pool were conducted in 160-ml serum bottles by adding 1 g of mat material along with 10 ml of filter-sterilized water from Obsidian Pool containing 10 mM $NaH^{13}CO_3$ or $NaH^{12}CO_3$. The serum bottles were sealed and flushed with N_2 to remove CO_2, and then O_2 was added to 13%. After the addition of H_2 to 1,000 ppm, the serum bottles were incubated in the dark without shaking at 37°C. The headspace H_2 concentration was monitored, and at the end of the experiment the mats were removed and centrifuged to remove water; the pellets were stored at −80°C.

Headspace analysis. Hydrogen content in the headspace was determined by gas chromatography (6850 Network GC system; Agilent Technologies) with a thermal conductivity detector and an HP PLOT MoleSieve 30-m by 0.53-mm by 25-μm column, a detector temperature of 250°C, an oven temperature of 50°C, and argon carrier gas at 3.3 ml min^{-1}. The limit of detection was 4 nmol H_2 ml^{-1}.

Isopycnic centrifugation and gradient fractionation. DNA was extracted from frozen samples using the MoBio (Carlsbad, CA) PowerSoil DNA extraction kit according to the manufacturer's directions. Extracted DNA (1 μg) was added to a CsCl solution in Tris-EDTA (TE) buffer (pH = 8.0) to a final volume of 2.0 ml and an average density of 1.729 g ml^{-1}. The ultracentrifugation tubes were sealed and centrifuged in a Sorvall S120-VT rotor at 190,000 g_{av} (where g_{av} is the average relative centrifugal field, which is calculated using the average radius of rotation) and 20°C for at least 60 h. The CsCl gradients were fractionated from bottom to top into 150-μl fractions by displacing the gradient with sterile water from the top of the tube with a syringe pump, and the density of each fraction was determined by measuring the refractive index of a subsample using an AR200 digital refractometer (Leica Microsystems). The DNA in each fraction was precipitated and resuspended as described previously (34). DNA from gradient fractions was stored at −80°C.

Denaturing gradient gel electrophoresis. Amplicons from gradient fractions were generated with the primers 341f-GC and 534r as described previously (49). Denaturing gradient gel electrophoresis (DGGE) was performed using the DGGEEK-2001 system (C.B.S. Scientific, Solana Beach, CA). PCR products (30 μl) were loaded on 8% (vol/vol) polyacrylamide gels with a 30 to 70% denaturant gradient (49), and electrophoresis was carried out at 60°C for 16 h at 70 V. Gels were imaged after staining with GelRed (Biotium, Inc., Hayward, CA) for 1 h. Selected bands were excised and reamplified with the primers 341f and 534r.

PCR, cloning, and sequencing. PCR amplification of 16S rRNA genes from gradient fractions was done using universal bacterial primers 8f and 1492r. The *hyd*B-like gene was amplified as described previously (12). PCR products were ligated into the vector pGEM-T Easy (Promega) by following the manufacturer's recommended protocol. Following transformation of plasmids into host cells, colonies were selected by blue/white screening. Cloned 16S rRNA genes were screened with DGGE by using the amplicons produced with vector-specific primers as the DNA template for nested PCR with the DGGE primer set 341f-GC and 534r. Selected clones that comigrated with DGGE bands from gradient fractions were sequenced using vector-specific primers by Genewiz, Inc. (South Plainfield, NJ). Raw sequence data were assembled into full-length sequences using the software program SEQMAN II (DNAStar, Inc., Madison, WI) and analyzed using nucleotide BLAST.

Nucleotide sequence accession numbers. All sequences were deposited at GenBank with accession numbers JF304432 to JF304447 for 16S rRNA gene sequences and JF304448 to JF304461 for *hydB* gene sequences.

RESULTS

SIP of knallgas bacteria. To identify knallgas bacteria using SIP, microcosms were established with soil from the rhizosphere of clover and vetch plants, and a microbial mat was collected from Obsidian Pool. The headspace hydrogen concentration was monitored to determine whether H_2 was consumed. When H_2 was provided at an initial concentration of 800 ppm, the microbial community in the clover soil oxidized H_2 much more rapidly than the community in the vetch soil (Fig. 1A). The maximum H_2 uptake rates observed for the clover soil under $^{12}CO_2$ plus H_2 or $^{13}CO_2$ plus H_2 were 0.50 μmol H_2 h^{-1} g $soil^{-1}$ and 0.46 μmol H_2 h^{-1} g $soil^{-1}$, respectively. The corresponding rates for the vetch soil were 0.21 μmol H_2 h^{-1} g $soil^{-1}$ and 0.17 μmol H_2 h^{-1} g $soil^{-1}$, respectively. Under an initial H_2 concentration of 1,000 ppm, the microbial mat samples from Obsidian Pool consumed H_2 steadily during the 38-h incubation (Fig. 1B), with both the ^{12}C plus H_2 and ^{13}C plus H_2 treatments consuming H_2 at a rate of 0.20 μmol H_2 h^{-1} g $sample^{-1}$ during the final 18 h. The H_2

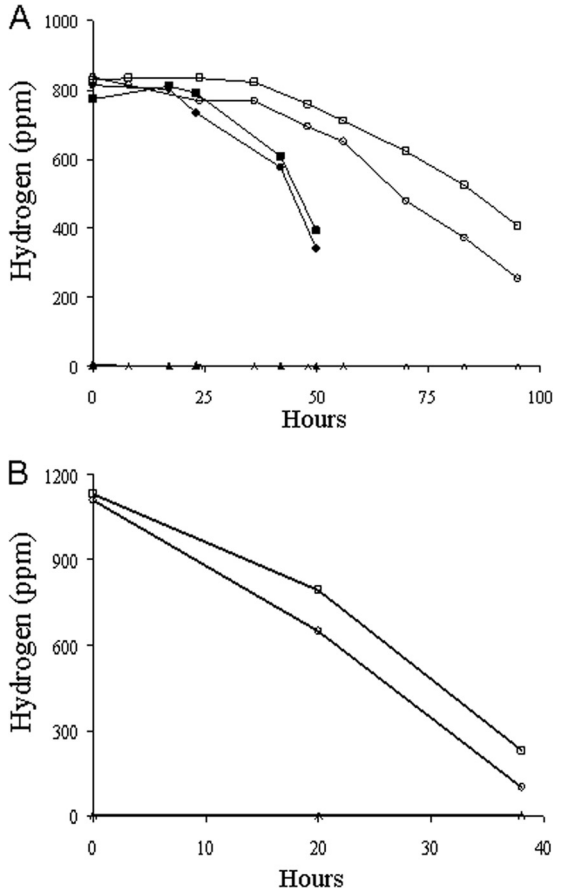

FIG. 1. Analysis of the hydrogen concentration during incubations of clover rhizosphere soil (filled symbols) or vetch rhizosphere soil (open symbols) (A) or a microbial mat from Obsidian Pool (B). The data points represent the mean hydrogen concentrations for duplicate measurements from the $^{13}CO_2$ without H_2 (triangles), $^{12}CO_2$ with H_2 (circles), or $^{13}CO_2$ with H_2 (squares) treatment.

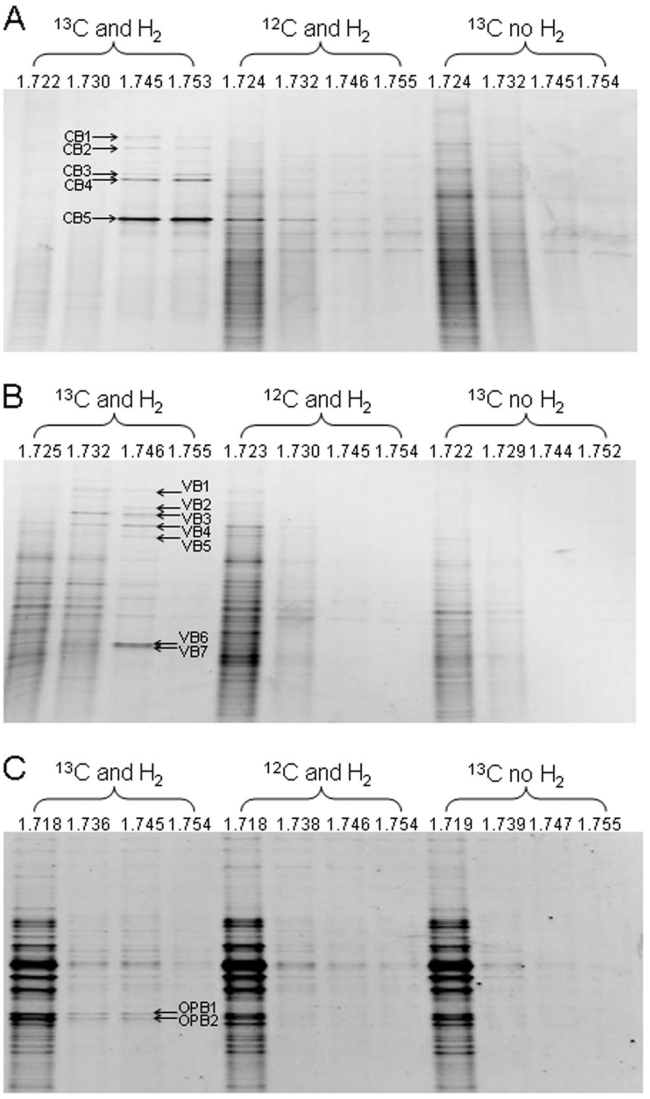

FIG. 2. DGGE profiles of 16S rRNA genes amplified from CsCl gradient fractions following isopycnic centrifugation of DNA extracted from clover rhizosphere soil (A), vetch rhizosphere soil (B), or a microbial mat from Obsidian Pool (C). The value above each lane indicates the density (g ml^{-1}) of that fraction.

uptake indicated that H_2-oxidizing bacteria were active in all three of the sampled ecosystems, and the increasing rates suggest growth of the populations, particularly in the soils.

Community profiles of density-resolved DNA. To determine whether incorporation of the ^{13}C label accompanied the H_2 uptake, 16S rRNA gene profiles from CsCl gradient fractions were compared by DGGE (Fig. 2). In all samples and treatments, there was a complex 16S rRNA gene profile in the less-dense fractions (1.718 to 1.732 g ml^{-1}), which contain the unlabeled DNA. In contrast, in the fractions with a density of 1.736 to 1.754 g ml^{-1}, no bands or only faint bands were detected when samples were incubated without H_2 or with $^{12}CO_2$ or [^{12}C]bicarbonate. Unique 16S rRNA gene profiles were only detected in the dense fractions when samples were given both $^{13}CO_2$ and H_2, indicating that autotrophic growth on H_2 occurred during the incubations. The bands that are present in the dense fractions of the ^{13}C plus H_2 treatment but are absent from comparable fractions in the control treatments represent autotrophic H_2-oxidizing bacteria.

Identification of knallgas bacteria. To identify which bacteria incorporated the $^{13}CO_2$, selected DGGE bands present in "heavy" fractions were sequenced along with cloned 16S rRNA genes that comigrated with the selected DGGE bands. The identified bacteria are listed in Table 1.

Five betaproteobacteria that incorporated $^{13}CO_2$ were identified in the clover rhizosphere soil. The strongest band in the heavy-fraction DGGE profile, CB5, was also the most abundant sequence in the clone library (25 of 48 clones). The 16S rRNA gene sequence shares 97% identity with that of *Aquincola tertiaricarbonis* L10, an isolate from methyl *tert*-butyl ether (MTBE)-contaminated groundwater that is capable of growing on *tert*-butanol but has not been reported to grow using H_2 (32). The 16S rRNA gene in band CB4 shares 99% identity with that of *Pelomonas saccharophila*, which is known to contain H_2-oxidizing strains. Other strains were most closely related to *Methylibium petroleiphilum*, *Ideonella dechloratans*, and *Aquaspirillum arcticum*. Two knallgas bacteria were isolated from the clover rhizosphere, and both are closely related to the

TABLE 1. Phylogenetic affiliation of partial bacterial 16S rRNA gene sequences corresponding to prominent bands identified by DGGE and isolated bacteria[a]

Sample source	DGGE band	Isolate	Closest relative[b]	% Identity	
				Band	Isolate
White clover	CB1		*Aquaspirillum arcticum* (AB074523)	96	
	CB2		*Ideonella dechloratans* (GU168990)	97	
	CB3		*Methylibium petroleiphilum* (AF176594)	98	
	CB4	C92	*Pelomonas saccharophila* (AM501428)	99	98
	CB5	C93	*Aquincola tertiaricarbonis* (DQ656489)	97	97
Hairy vetch	VB1	VB55	*Variovorax paradoxus* (AB552859)	99	99
	VB3		*Polaromonas rhizosphaerae* (EF127651)	99	
	VB4		*Methylibium fulvum* (AB245357)	97	
	VB5		*Methylibium fulvum* (AB245357)	98	
	VB6		*Aeromicrobium ginsengisoli* (FR682667)	98	
	VB7		*Aeromicrobium ginsengisoli* (AB245395)	97	
Obsidian Pool	OPB1		*Spirochaeta caldaria* (EU580141)	99	
	OPB2		*Magnetospirillum bellicus* (EF405824)	98	

[a] Except for bands VB6 and OPB1, cloned sequences matching directly sequenced bands were used to determine the closest relatives.
[b] GenBank accession no. is given in parentheses.

H_2-oxidizing bacteria identified by SIP. The 16S rRNA genes of isolates C92 and C93 share 99% and 99.7% sequence identity to bands CB4 and CB5, respectively.

The H_2-oxidizing bacteria identified from the vetch rhizosphere soil were primarily betaproteobacteria, as well. The only band that corresponded to a species known to contain H_2-oxidizing bacteria was VB1, which shares 99% 16S rRNA gene sequence identity with *Variovorax paradoxus*. The sole knallgas bacterium isolated from the vetch rhizosphere possesses a 16S rRNA gene with 99.9% sequence identity to band VB1. The predominant cloned sequences from the vetch soil corresponded to bands VB3 (13 of 48) and VB4 (12 of 48) and were closely related to *Polaromonas rhizosphaerae* and *Methylibium fulvum*, respectively. The closest relative of band VB5 was also a *Methylibium fulvum* species. The cloned sequence matching band VB7 shares 97% identity with *Aeromicrobium ginsengisoli*, an actinomycete isolated from a ginseng field (25). No cloned sequence matched band VB6, but the sequence of the extracted DGGE band shares 98% identity with *Aeromicrobium ginsengisoli*. Hydrogen oxidation has not been reported previously for *Aeromicrobium* species.

Two bands were enriched in ^{13}C from the Obsidian Pool microbial mat. Based on 16S rRNA gene similarity, the organism represented by band OPB2 is a member of the alphaproteobacteria and is closely related to *Magnetospirillum bellicus* strain VDY, a strain isolated for its ability to reduce perchlorate (52). The sequence of the band OPB1 shares 99% sequence identity with that of *Spirochaeta caldaria* strain DSMZ7334. A clone matching band OPB1 was not obtained. The organisms represented by bands OPB1 and OPB2 are closely related to bacteria known to oxidize H_2 under anaerobic conditions (37, 52), which suggests that anaerobic conditions developed in the mat during the SIP incubation even though the O_2 concentration in the headspace was never below 11%.

Hydrogen concentration threshold for detection by SIP. To determine the potential application of SIP to detect knallgas bacteria with low H_2 thresholds, SIP incubations with the clover and vetch soils were conducted under H_2 concentrations of 500, 250, and 100 ppm. Although H_2 uptake was observed at all concentrations, H_2 uptake kinetics differed between the two soils (Fig. 3). At 500 ppm, the clover soil consumed H_2 with a maximum rate of uptake of 0.52 μmol H_2 h^{-1} $g\ soil^{-1}$, whereas the maximum uptake rate for the vetch soil was 0.28 μmol H_2 h^{-1} $g\ soil^{-1}$. At 100 ppm H_2, the maximum uptake rates were similar between the clover and vetch soil, 0.07 and 0.08 μmol H_2 h^{-1} $g\ soil^{-1}$, respectively. However, the vetch soil displayed a much shorter lag time for H_2 consumption and completely removed H_2 from the headspace, suggesting that knallgas bacteria from the vetch soil are better adapted for H_2 uptake at lower concentrations.

DGGE analysis of CsCl gradient fractions revealed that the majority of bacteria detected by SIP when the soils were incubated under 250 to 500 ppm H_2 were not detected at 100 ppm H_2 (Fig. 4). When the clover soil was incubated under 100 ppm H_2, only faint ^{13}C-DNA bands were detected, indicating that the indigenous knallgas bacteria grew poorly when the H_2 concentration was below 250 ppm. When the vetch soil was incubated with 100 ppm H_2, only bands VB6 and VB7 were detected. The detection of the *Aeromicrobium* species and the absence of the betaproteobacteria at 100 ppm H_2 suggest that the actinomycetes had a lower H_2 threshold for chemolithotrophic growth.

Amplification of *hydB* hydrogenase genes from ^{13}C-labeled DNA. Because the actinomycetes detected in the vetch soil appear to grow well even at the lowest H_2 concentration tested, primers designed to amplify the *hydB*-like gene were used to test for the presence of the putative high-affinity hydrogenase that has been found in species of *Streptomyces* (12). An ~1.4-kb *hydB* homolog was detected in the heavy fractions (1.737 to 1.755 g ml^{-1}) from both the vetch and clover soils that were treated with H_2 and $^{13}CO_2$ (Fig. 5); no amplicons were detected in the heavy fractions from soils incubated with $^{12}CO_2$. Nontarget amplicons that are shorter than the expected 1.4 kb were obtained from the light fractions of both the vetch

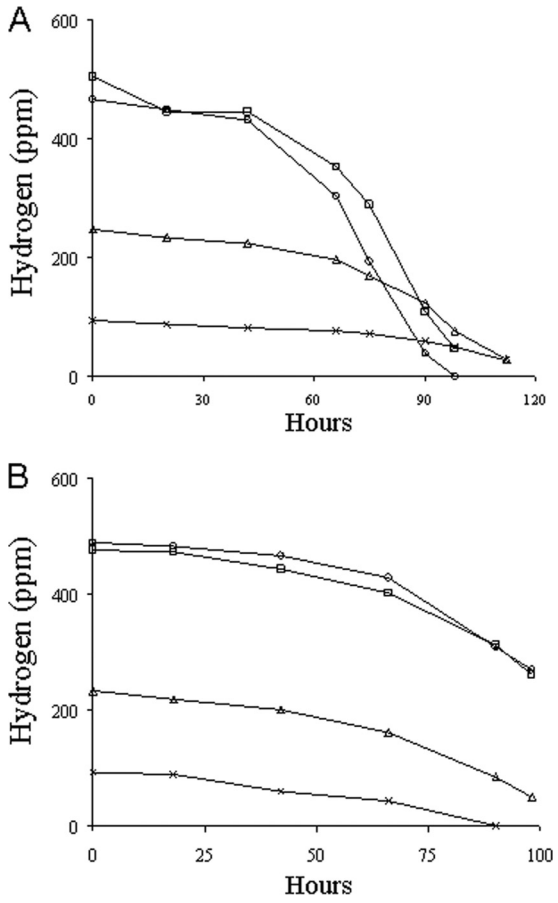

FIG. 3. Analysis of the hydrogen concentration during incubations of clover rhizosphere soil (A) or vetch rhizosphere soil (B). The data points represent the mean hydrogen concentrations for duplicate measurements from the $^{12}CO_2$ with 500 ppm H_2 (○), $^{13}CO_2$ with 500 ppm H_2 (□), $^{13}CO_2$ with 250 ppm H_2 (△), and $^{13}CO_2$ with 100-ppm H_2 (×) treatments.

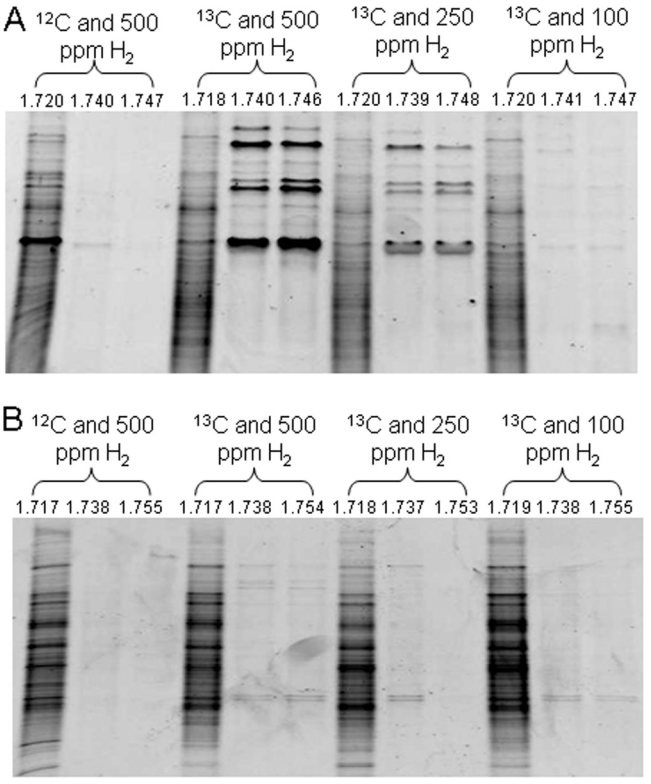

FIG. 4. DGGE profiles of 16S rRNA genes amplified from CsCl gradient fractions following isopycnic centrifugation of DNA extracted from clover rhizosphere soil (A) or vetch rhizosphere soil (B) incubated under 500, 250, or 100 ppm H_2. The value above each lane indicates the density (g ml^{-1}) of that fraction.

and clover soils (1.717 to 1.719 g ml^{-1}). A *hydB* homolog could not be amplified from the knallgas bacterium isolates.

Phylogenetic analysis of the predicted amino sequences of *hydB* homologs that were cloned from the two soils revealed that the hydrogenases clustered according to the soil from which they were amplified (Fig. 6). Hydrogenases from the clover soil formed a clade with the NiFe hydrogenases found in isolated *Streptomyces* species that can oxidize tropospheric H_2, whereas the hydrogenases from the vetch soil formed a clade with hydrogenases that are primarily associated with uncultured bacteria and the actinomycete *Conexibacter woesei*. Although it is clear that the putative high-affinity hydrogenase is present in the bacteria active during the SIP incubations, it remains unclear which organisms harbor the gene.

DISCUSSION

The results indicate that knallgas bacteria can be identified by SIP. SIP provides insight into the metabolic function of uncultivated populations (39), and in this study, three knallgas bacteria were identified by both cultivation and SIP, while eight additional H_2-oxidizing autotrophs were identified by SIP alone. There is minor variation between the 16S gene sequences of the isolates and the corresponding sequences obtained by SIP. However, variation within a phylotype, or even a strain, is not unexpected (1, 28), and the isolates and bacteria identified by SIP are clearly members of the same ecotype. Because we isolated knallgas bacteria from serial dilutions, the cultured isolates represent only the most abundant knallgas bacteria. It is also possible that many of the knallgas bacteria that were detected by SIP could not grow in isolation under the culture conditions we provided.

The bacteria identified by SIP are closely related to both previously described knallgas bacteria and bacteria that were not known to grow autotrophically using H_2. The detection of three knallgas bacteria by both cultivation and SIP supports the conclusion that the uncultivated bacteria detected by SIP were knallgas bacteria as well. Furthermore, although chemolithotrophic growth on H_2 has not been reported for most of the bacteria detected by SIP, many are from genera with known H_2-oxidizing bacteria. For example, growth on H_2 has not been documented in *P. rhizosphaerae*, but both *Polaromonas hydrogenivorans* and *Polaromonas naphthalenivorans* CJ2 can grow chemolithotrophically with H_2 (48). However, because we cannot dismiss the possibility that anaerobic niches existed within the soil during the incubations, we cannot conclude with absolute certainty that all of the bacteria identified by SIP were aerobic H_2-oxidizing bacteria.

The observed range of hydrogen uptake rates during SIP

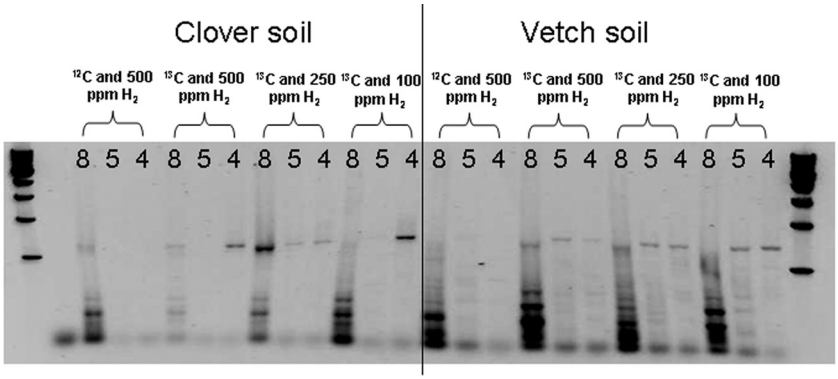

FIG. 5. Analysis of PCR products obtained from clover and vetch soil with primers targeting the *hydB*-like gene by gel electrophoresis (negative image). The number above each lane indicates which gradient fraction the template was from: 8, density of 1.717 to 1.720 g ml^{-1}; 5, density of 1.737 to 1.741 g ml^{-1}; 4, density of 1.745 to 1.748 g ml^{-1}. A 1-kb DNA step ladder was used as the marker.

incubations were comparable to previously published V_{max} values, which range from 0.01 to 0.4 μmol H_2 h^{-1} g $soil^{-1}$, for the oxidation of H_2 by soil (8). However, there were significant differences in H_2 uptake kinetics between the two soils. When the H_2 concentration was above 100 ppm, the difference in H_2 uptake by the microbes in the clover and vetch rhizosphere soils was likely due to faster growth of the knallgas bacteria in the clover soil. The difference in H_2 uptake by the microbes in the two soils at l00 ppm H_2 can be attributed in part to the presence of the actinomycetes in the vetch soil, because DGGE analysis revealed that incorporation of $^{13}CO_2$ at 100 ppm H_2 was limited to bands VB6 and VB7.

The detection of actinomycetes growing autotrophically at 100 ppm H_2 provides further evidence that actinomycetes play a role in H_2 oxidation in soil at ecologically relevant concentrations. *Streptomyces*, *Pseudonocardia*, and *Mycobacterium* species were previously implicated as H_2 users in soil exposed to H_2 and in soil surrounding the root nodules of field-grown soybeans based on changes in T-RFLP profiles (41). *Mycobacterium smegmatis* was identified as a high-affinity H_2 oxidizer, with an uptake threshold of 1 ppm (26). In addition, recently isolated *Streptomyces* species that harbor a putative high-affinity NiFe hydrogenase can oxidize tropospheric H_2 and may be significant in the H_2 biogeochemical cycle (12). More work is needed to determine the role *Aeromicrobium* species play in the oxidation of H_2 in soil.

Initially, we attempted to amplify the *hydB* homolog because it was previously associated with actinomycetes. We hypothesized that the *hydB* homolog might be responsible for the apparent advantage of *Aeromicrobium* species in the vetch soil at lower H_2 concentrations. Although originally detected in *Streptomyces* species, the *hydB* homolog from the vetch soil formed a clade with hydrogenases that are associated with uncultured bacteria and the actinomycete *Conexibacter woesei*, suggesting *hydB* is more widespread in the actinomycete group. Because a *hydB* homolog was detected in the ^{13}C-labeled DNA from both soils, additional study is needed to link the *hydB* homolog to specific knallgas bacteria identified by SIP. In order to establish a connection between the *Aeromicrobium* species and the *hydB* homolog, it will be necessary to isolate the strain or observe association of the VB6 or VB7 16S rRNA gene with the *hydB* homolog, perhaps by using GeneFISH (38). The detection of hydrogenase genes in the ^{13}C-labeled DNA suggests that cultivation-independent detection of O_2-tolerant hydrogenases (20) could be accomplished by designing SIP experiments that select for O_2-tolerant knallgas bacteria.

The bacteria identified in the Obsidian Pool microbial mat appear to be anaerobic H_2-oxidizing bacteria rather than knallgas bacteria. To minimize disturbance of the microbial mat, the samples were not shaken; thus, it is likely that anaerobic microenvironments developed. Band OPB1 is likely from a spirochete, and acetogenic growth on H_2 and CO_2 has been reported for spirochetes (37), while aerobic H_2 oxidation has not. Although preliminary, this may be the first example of a hydrogenotrophic acetogen detected by SIP. Monosaccharide-consuming and propionate-oxidizing acetogens have been detected previously (35, 43), but further work is needed to determine whether SIP could be a useful tool for the study of hydrogenotrophic acetogens. The organism corresponding to band OPB2 is closely related to *M. bellicus* strain VDY, which can grow chemolithotrophically with H_2 as an electron donor using perchlorate as an electron acceptor (52), and although strain VDY can grow aerobically, aerobic growth on H_2 has not been reported. Therefore, our results cannot conclusively determine whether the growth of OPB2 was aerobic or anaerobic. The results support the conclusion that SIP enabled the detection of bacteria that incorporated the ^{13}C-labeled substrate, and the incubation conditions determine whether aerobic or anaerobic H_2-oxidizing chemolithotrophs are detected.

Because many knallgas bacteria are facultative autotrophs, SIP is not appropriate for detecting knallgas bacteria in samples with high levels of organic carbon. Transfer of carbon from cyanobacteria to heterotrophic bacteria has been demonstrated with nanoSIMS (5), and organic carbon provided by cyanobacteria in microbial mats could repress autotrophic growth by knallgas bacteria. Furthermore, H_2 oxidation is not limited to knallgas bacteria, and uptake hydrogenase activity in heterotrophic N_2-fixing bacteria or cyanobacteria can compete with knallgas bacteria for H_2 during SIP incubations. During our investigations, cyanobacterium-dominated samples frequently consumed H_2 rapidly during SIP incubations, but ^{13}C-labeled DNA was rarely detected. Some cyanobacteria are able to use H_2 as an electron donor to fix CO_2 in a process called photoreduction (6, 17), but CO_2 fixation and growth with H_2 as

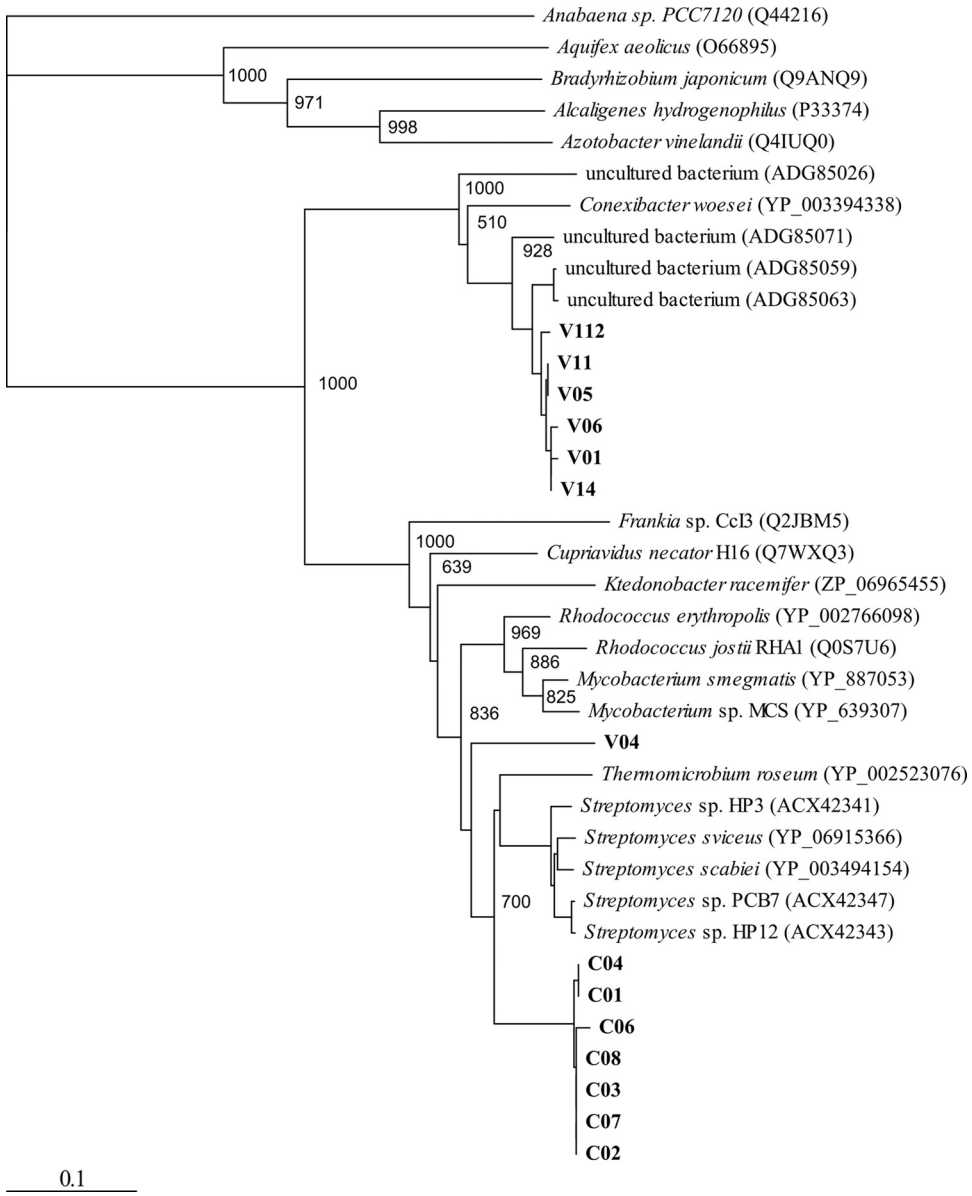

FIG. 6. Phylogenetic tree of partial amino acid sequences translated from *hydB*-like gene sequences that were cloned from the "heavy" SIP fractions from vetch and clover soil (bold) and gene sequences for large subunits of NiFe hydrogenase available in public databases. Cloned sequences with names beginning with C were amplified from the clover soil, and those beginning with V were amplified from the vetch soil. The scale bar represents 10% sequence divergence.

an energy source are not expected to be significant for cyanobacteria incubated in the dark (6), and we did not observe ^{13}C-labeled cyanobacterial DNA.

The methods described here should be applicable to many other ecosystems, such as soil, marine, and geothermal systems. For example, SIP could complement studies of the interaction between plants and H_2-oxidizing bacteria and reveal whether certain plants increase carbon fixation in soils by promoting the growth of knallgas bacteria. Further work is needed to determine whether knallgas bacteria that oxidize H_2 at tropospheric levels can be detected by SIP, though it is likely that longer incubations will be necessary, because ^{13}C-DNA was not detected in the controls incubated under ambient air without additional H_2.

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